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(71) Applicant: **Mycogen Corporation**
5451 Oberlin Drive
San Diego, CA 92121(US)

(72) Inventor: **Wilcox, Edward R.**
5 Lopa Court
North Potomac, Maryland 20878(US)

(74) Representative: **Perry, Robert Edward et al**
GILL JENNINGS & EVERY 53-64 Chancery Laneane
London WC2A 1HN(GB)

(54) **Improved control of expression of heterologous genes from lac operated promoters.**

(57) The invention is a process for controlling the expression of heterologous genes from lac -operated promoters by removing the CAP binding site and lac promoter from the lac operon. Illustrated is the fusion of the lacZ , Y , and A genes of the lac operon to the 3' end of the lacI structural gene. This elimination of the natural regulatory elements of the lac operon results, advantageously, in the production of lac operon gene products in a constitutive mode from the lacI promoter.

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IMPROVED CONTROL OF EXPRESSION OF HETEROLOGOUS GENES FROM LAC OPERATED PROMOTERS

Background of the Invention

The lactose (lac) operon consists of three protein products under the control of a lac promoter-operator. These gene products are β-galactosidase (Z) permease (Y), and thiogalactoside transacetylase (A). The protein product of the lacI transcript (repressor), an independent gene product, interacts with the operator of the lac operon and keeps synthesis off until allolactose (1,6-0-β-D-galactopyranosyl-D-glucose), a product of the β-galatosidase reaction, accumulates in the cell and binds to the repressor. The allolactose repressor complex has a changed conformation, allowing the repressor to be displaced from the operator. RNA transcription then begins from the lac promoter (Beckwith, J. [1987] In *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology . Vol. 2, Neidhardt, F.C., Editor in Chief, American Society for Microbiology, Washington, D.C.).

A few molecules of the lac operon transcript are present in E. coli , even in the absence of lactose. Hence, permease and β-galactosidase are always present, at least at a low level. Allolactose, the natural inducer of the operon, is made in the cell when lactose (1,4-0-β-D-galactopyanosyl-D-glucose) enters the cell by the permease reaction and is converted through transgalactosidation by β-galactosidase into allolactose (Freifelder, D., [1987] Molecular Biology . Jones and Bartlett Publishers, Inc., Portola Valley, CA; Beckwith, supra). It was calculated that greater than 20% of the lactose acted upon by β-galactosidase is converted to allolactose (Jobe, A., Bourgeois, S., [1972] J. Mol. Biol. 69 :397-408). The majority of the remaining lactose is converted to glucose and galactose. Allolactose is a better substrate for β-galactosidase than lactose (Jobe and Bourgeois, supra , and is itself rapidly converted to glucose and galactose.

Another control element of the lac operon is catabolite repression. In the presence of glucose the cell is able to repress many operons. For example the lac operon is only transcribed at 2% of its maximum level in the presence of glucose (Beckwith, supra).

When several β-galactosides were compared for their ability to induce the lac operon in vivo , lactose was found to act as a very poor effector molecule. The synthetic non-metabolized β-galactoside, isopropyl-β-D-thiogalactoside (IPTG), was found to induce the lactose operon 5 times better than lactose (Monod, J., G. Cohen-Bazire and M. Cohn [1951] Biochim. Biophys. Acta. 7 :585-599). Yet, allolactose itself is as good an inducer of the operon as IPTG (Muller-Hill, B., H.V. Rickenberg and K Wallenfels [1964] J. Mol. Biol. 10 :303-318; Jobe and Bourgeois, supra).

Given the efficiency of lactose conversion to allolactose, one might expect lactose to work very well as an inducer of the lac operon. However, prior to the present disclosure, this has never occurred in practice (Monod et al., supra).

The current way to control the expression of heterologous genes from the lac promoter or lac concensus promoters such as tac (Rezinkoff, W.S. and W.R. McClure [1986] Maximizing Gene Expression , W. Reznikoff and L. Gold, eds., Butterworth Publishers, Stoneham, MA) is to have enough lac repressor present in the cell, so that transcription from the tac promotor is off until IPTG or another proper inducing β-galactoside is added to the cell. Although IPTG is the current inducer of choice, it is expensive and has been labeled a potential carcinogen. Thus, there is a need to replace IPTG in commercial systems where control of the expression of heterologous genes from lac operated promoters is used.

Brief Summary of the Invention

The subject invention concerns an improved method for controlling the expression of heterologous genes from lac operated promoters. Specifically, the subject invention concerns a method for controlling the expression of heterologous genes from lac operated promoters which comprises removing the CAP binding site and lac promoter/operator from the lac operon. The subject invention is exemplified herein by use of a novel recombinant DNA construct comprising the lacZ , Y and A genes of the lac operon, to control the expression of heterologous genes from lac operated promoters. The lacZ , Y , and A genes of the lac operon were fused to the 3′ end of the lacI structural gene, thereby eliminating all of the natural regulatory elements of the lac operon. By doing this, lac operon gene products are produced in a constitutive mode from the lacI promoter and are not responsive to catabolite repression or to allolactose induction. When lactose is added, the constitutively synthesized β-galactosidase converts as much as 20% to allolactose,

which in turn derepresses the tac promoter and any associated heterologous gene. As long as the allolactose concentration in the cell is above 10^{-5}M the tac promoter provides high expression of the protein of interest. The complete DNA sequence of the transcriptional fusion of the lacI gene to the lacZ , Y , and A genes of the subject invention is as shown in Table 1.

The novel lacIZYA operon (Table 1) can be inserted in any plasmid, or it can be inserted into any microbial chromosome to improve control of heterologous gene expression from a lac operated promoter (as long as a promoter is present to drive lacIZYA transcription). It will be apparent to a person skilled in the art that other constructs can be used to fuse the genes.

The lac operator, which has very high affinity for the lacI repressor (K_{DNA} = 2-5x10^{13} M^{-1}), is a sequence 21 b.p. long as follows; AATTGTGAGCGGATAACAATT (Barkley, M.D. and S. Bourgeois [1978] In The Operon Miller, J.H. and W.S. Reznikoff, eds. Cold Spring Harbor). Many mutations within the lac operator exist which have higher or lower affinities to the lacI repressor (Barkley and Bourgeois, supra ; Sadler, J.R. et al. [1983] Proc. Natl. Acad. Sci. USA 80 :6785-6789; Simons, A. et al. [1984] Proc. Natl. Acad. Sci. USA 81 :1624-1628). Many mutations exist within the lacI repressor that allow for greater affinity to the U operator with no significant effect on derepression (Barkley and Bourgeois, supra). Using any of the many different combinations of lac operator and lacI repressor, the control of heterologous gene expression is apparent to a person skilled in the art. Many different promoters (eukaryotic as well as prokaryotic) have been placed under lac operator regulation (Yansura, D.G. and D.J. Henner [1984] Proc. Natl. Acad. Sci. USA 81 :439-443; Herrin Jr., G.L and G.N. Bennett [1984] Gene 32 :349-356; Deuschle, U., et al. [1986] Proc. Natl. Acad. Sci. USA 83 :4134-4137; Hu, M.C-T. and N. Davidson [1988] Gene 62 :301-314; Figge, J., et al. [1988] Cell 52 :713-722). The subject invention is designed to improve lactose induction from such lac operated promoters.

This invention enables cells to make enough lacZ and Y protein to efficiently take up lactose and convert it to glucose and galactose, as well as to the true lac inducer, allolactose. In addition, the invention enables the cell to synthesize enough repressor (lacI protein) to bind to the lac operator upstream from a heterologous gene, and keep heterologous gene expression off in the absence of inducer. With excess lactose present in the medium, the cell can accumulate sufficient concentrations of allolactose for efficient derepression of the lac operator.

Brief Description of the Drawings

Figure 1 - pMYC 2005, LacI and Z operon fusion

A synthetic fragment of DNA was cloned into pUC18 to replace the normal sequence found at Hin d III-403 to Hae II-524. This synthetic sequence removes the lac promoter and operator sequences and replaces them with a Shine-Dalgarno site.

Figure 2 - pMYC 2101, construction of the lacIZYA operon

Three fragments of DNA were ligated and then introduced by transformation into MC1061 (Casadaban, M. and S. Cohen [1980] J. Mol. Biol. 138 :179-207) to construct the plasmid pMYC2101. The first fragment came from pMYC2005 as a Hin dIII-403 to Eae I-485 piece, the second fragment came from pMC9 as an Eco RI-1 to Eae I-1103 piece and the final fragment comes from pSKS107 as a 9889 bp Hin dIII-31 to Eco RI-1 piece. Once constructed, the Eco RI and Sal I ends were converted to Bam HI and Bgl II ends, respectively, using oligonucleotide linkers, creating pMYC2101-B.

Figure 3 - pMYC467, a tac promoted toxin-containing plasmid

The tac promoted toxin gene found in pMYC436 (NRRL deposit no. B-18292) was cloned as a 4.5 Kbp Bam HI to Pst I fragment in the vector pTJS260. Not all the sequence of pTJS260 or of the 3′ flanking sequences of the toxin genes are available, hence some restriction sites are approximated in Figures 3, 4, 5 and 6.

Figure 4 - pMYC471, a lactose inducible plasmid

The lacIZYA operon was cloned into the Bam HI site of pMYC467. The resulting plasmid, pMYC471, was then induced by transformation into MB101, a P. fluorescens .

Figure 5 - pMYC485, an alternate lactose inducible plasmid

A $lacI^{Q}ZYA$ operon was constructed by replacing lacI with $lacI^{Q}$ as a 638 bp Bam HI to Apa I fragment. The $lacI^{Q}ZYA$ operon was then cloned into the Bam HI site of pMYC467. The resulting plasmid, pMYC485, was tested in MB101 for lactose inducibility. The $lacI^{Q}ZYA$ operon is shown in Table 1 wherein base 16 is a T instead of a C as shown in the table.

Figure 6 - pMYC1611, a coleopteran toxin-lactose inducible plasmid

The plasmid pMYC471 was cut with Bam HI (at position 12103) and Kpn I (at position 7933) to remove the tac -promoted lepidopteran-active toxin and then both ends were filled in with T4 DNA polymerase, resulting in blunt termini. The tac promoted toxin gene disclosed in U.S. Patent 4,771,131, was inserted into the above DNA as a blunt-ended Sca I (of pBR322) to filled-in Hin dIII fragment (at the 3′ end of the coleopteran-active sequence). The 5′ end of the tac promoter had been previously inserted by blunt-end ligation into the Eco RI site of pBR322.

Detailed Disclosure of the Invention

Upon removal of the CAP binding site and lac promoter/operator from the lac operon, there is realized an improved control of expression of a heterologous gene. Exemplified herein is the insertion of a lacIZYA fusion construct into a plasmid containing a tac -promoted gene encoding a lepidopteran insect toxin. The plasmid (pMYC47I) was then used to transform Pseudomonas fluorescens , which was designated Pseudomonas fluorescens MR471 (pMYC471).

One element of plasmid pMYC471 provides its host with constitutive levels of lac operon gene products. For this purpose, the operon could be fused to any of a variety of available constitutive promoters. In this instance, it was fused to the lacI promoter. In addition, a new construct containing a lacI^{Q} promoter, instead of the lacI promoter, was made (pMYC485). This new construct provides higher levels of the repressor as well as of lac operon gene products. The plasmid, pMYC4BS, functions like pMYC471.

As a demonstration that lactose inducibility is not dependent on the specific gene under tac promoter control, another gene encoding a coleopteran-active toxin was cloned into the pMYC471 vector replacing the lepidopteran-active toxin gene, resulting in pMYC1161. Pseudomonas fluorescens strain MB101 carrying this new plasmid also permits lactose induction of toxin expression. This will hold true for any gene under control of a lac operated promoter.

Another aspect of this fusion is that the lac operon is no longer regulated by the normal control elements associated with lactose. This means that synthesis of lac operon ZYA gene products in these constructs (pMYC471, pMYC485, and pMYC1161) is constitutive and occurs independently of the concentrations of lactose or glucose present in the cell. For example, in the presence or absence of IPTG or lactose, a new protein band corresponding to the mass of β-galactosidase is apparent on SDS-PAGE and the clones cleave X-gal, forming blue colonies under the same conditions.

For ease in construction, the lacA gene has been included on all the plasmids contining lacIZYA mentioned in this disclosure. The lacA gene product has no role (Freifelder, supra) in production of allolactose and can easily be removed from the lacIZYA operon. For example, there is a non- unique Afl II (CTTAAG) restriction enzyme site a the stop codon of the lacY gene. By using this AFI II restriction site, it is possible to remove the lacA gene. The lacIZY operon has the same lactose induction properties as those found in the lacIZYA operon.

The data of Table 2 demonstrate toxin production is regulated by lactose or IPTG. Although the amount of lactose used for induction is 10 to 20 fold higher than IPTG induction levels, the significantly reduced cost of lactose ($7.00 per kilo, Sigma Chemical Co.) compared to IPTG ($20,000 per kilo, Sigma Chemical Co.) make the former inducer highly economically advantageous. For maximum production levels the metabolized substrate, lactose, must be replenished during cell growth or the culture will decrease the synthesis of the tac -promoted gene.

Materials and Methods

Cloning and DNA manipulation techniques are described in Maniatis, T., E.F. Fritsch and J. Sambrook (1982) Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory Publishers, Cold Spring Harbor, New York.

A plasmid containing the lac operon, pSKS107, was received from Dr. M. Casadaban at the University of Chicago (Shapira, S.K, J. Chou, F.V. Richaud and M.J. Casadaban [1983] Gene 25 :71-82). The sequence of the lac operon is published (Kalnins, A., K Otto, U. Ruther and B. Muller-Hill [1983] EMBO J. 2 :593-597; Hediger, M.A, D.F. Johnson, D.P. Nierlich and I. Zabin [1985] Pro. Nat. Acad. Sci. 82 :6414-6418). The lacI gene, on a 1.7KB Eco RI restriction fragment cloned into pBR322, is available from the ATCC, as pMC9, catalogue no. 37195, 37196 and 37197. The sequence of the lacI gene is also published (Farabaugh, P.J. [1978] Nature 274 :765-769). The broad host range vector, pMMB22, was received from Dr. M.

Bagdasarian (Bagdasarian, M.M., E. Amann, R. Lurz, B. Ruckert and M Bagdasarian [1983] Gene 26 :273-282). A Hin dIII fragment of pMMB22, containing the lacI ^{Q} gene, was relinkered to Bam HI. The sequence of the lacI ^{Q} gene has also been published (Calos, M.P. [1978] Nature 274 :762-765). Analysis here is largely based on the published DNA sequences. Some of the critical portions of the constructs were confirmed by DNA sequencing, e.g., the lacI and lacI ^{Q} promoters and the fusion region between the lacI transcriptional unit and the lac operon. The vector, pUC18, is available from a variety of sources, such as catalogue no. 27-4949-01 of Pharmacia Corporation. The broad host range vector, pTJS260, is available from Dr. D.R. Helinski at the University of California, San Diego, La Jolla CA 92093 (Schmidhauser, T.J. [1986] Ph.D Thesis, UCSD).

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 - Fusions of the LacI Gene and Lac Operon.

Fusions of the lacI gene and lac operon were first carried out in pUC18. To do so, pUC18 was linearized with Hin dIII and then partially cut with Hae II. The DNA was resolved by electrophoresis on a 1.4% agarose gel. The 2402 bp band was eluted and ligated to a double-stranded synthetic DNA insert produced by β-cyanoester chemistry on an Applied Biosystems 380A oligonucleotide synthesizer. This sequence starts at BP 1073 of the lacI sequence (Farabaugh, P.J., supra) as seen in Table 1.

Hae II Eae I

CC AAT ACG CAA ACC GCC TCT CCC CGC GCG TTG GCC GAT
CGCGGG TTA TGC GTT TGG CGG AGA GGG GCG CGC AAC CGG CTA

Xho I

TCA TTA ATG CAA CTC GCA CGA CAG GTC TCG AGA CTG GAA AGC
AGT AAT TAC GTT GAG CGT GCT GTC CAG AGC TCT GAC CTT TCG

lacI Stop S/D lacZ Start Hind III

GGG CAG TGA GCGCTAGGAGGTAACTT ATG GAA
CCC GTC ACT CGCGATCCTCCATTGAA TAC CTTTCGA

The above synthetic sequence removes the Pvu II site normally found at BP 1123 of lacI . A new XhoI site was inserted near the 3′ end of the lacI gene (BP 1137). These changes were introduced for ease in identification of the new construct. No amino acid changes would occur as a result of the mutations introduced in making the base pair changes for the above two restriction sites. In this construct, the distance between the stop codon of lacI and the start codon of lacZ is 17 base pairs. This region contains a ribosome binding site (marked as S/D) such that a ribosome translating the lacI transcript will be able to continue synthesis of the lacZ gene product from the same transcript. A plasmid diagram of pMYC2005 is seen in Figure 1.

Example 2 - Confirmation of Sequence.

The synthetic portion of pMYC2005 was sequenced to validate its structure and was used in a three-piece ligation to tie the full lacI gene of pMC9 to the lac operon found in pSKS107 (Figure 2). After transformation, the lacI/Z fusion region was sequenced from a blue colony on an LB + X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase, a colorimeteric substrate for the presence of β-galactosidase) plate. Those restriction sites that were examined yielded fragments of the predicted sized (Figure 2). The lacI

promoter region was also confirmed by sequence analysis.

Example 3 - Cloning.

The Eco RI and Sal I ends of the lacIZYA construct (Figure 2) were respectively relinkered to yield Bam HI and Bgl II restriction sites. The lacIZYA operon was cloned into the unique Bam HI site of pMYC467 (Figure 3) yielding pMYC471 (Figure 4). The plasmid, pMYC467, contains the tac promoted lepidopteran toxin gene of pMYC436 (a cry IA(c)-like toxin gene [NRRL deposit no. B-18292] Adang, M.J. et al. [1985] Gene 36 :289-300) cloned into the broad host range vector, pTJS260. pMYC471 was introduced by transformation into P. flourescens MB101 and the resulting clone was designated MR471. MR471 was tested for the key elements of the resident pMYC471 plasmid. A functional repressor is synthesized because in the absence of IPTG or lactose no significant amount of toxin is produced (Table 1). The cells are blue when plated on X-gal because a functional β-galactosidase is present. A functional permease and β-galactosidase are present because the cells are able to induce the tac promoter in the presence of lactose (Table 1).

The subject invention can be used with any heterologous gene to control its expression from lac operated promoters. The expression product (protein) can be isolated from the culture medium of the producing microbe by means known in the art for isolating such a product from microbial cultures. Alternatively, a product which remains intracellular can be used in the form of the microbe itself, for example, as a biological insecticide. See U.S. patents 4,695,455 and 4,695,462 for such uses.

Table 1

T in lac I^Q

```
   1  GACACCATCG AATGGCGCAA AACCTTTCGC GGTATGGCAT GATAGCGCCC

                                start codon
                                lac I
  51  GGAAGAGAGT CAATTCAGGG TGGTGAATGT GAAACCAGTA ACGTTATACG
 101  ATGTCGCAGA GTATGCCGGT GTCTCTTATC AGACCGTTTC CCGCGTGGTG
 151  AACCAGGCCA GCCACGTTTC TGCGAAAACG CGGGAAAAAG TGGAAGCGGC
 201  GATGGCGGAG CTGAATTACA TTCCCAACCG CGTGGCACAA CAACTGGCGG
 251  GCAAACAGTC GTTGCTGATT GGCGTTGCCA CCTCCAGTCT GGCCCTGCAC
 301  GCGCCGTCGC AAATTGTCGC GGCGATTAAA TCTCGCGCCG ATCAACTGGG
 351  TGCCAGCGTG GTGGTGTCGA TGGTAGAACG AAGCGGCGTC GAAGCCTGTA
 401  AAGCGGCGGT GCACAATCTT CTCGCGCAAC GCGTCAGTGG GCTGATCATT
 451  AACTATCCGC TGGATGACCA GGATGCCATT GCTGTGGAAG CTGCCTGCAC
 501  TAATGTTCCG GCGTTATTTC TTGATGTCTC TGACCAGACA CCCATCAACA
 551  GTATTATTTT CTCCCATGAA GACGGTACGC GACTGGGCGT GGAGCATCTG
 601  GTCGCATTGG GTCACCAGCA AATCGCGCTG TTAGCGGGCC CATTAAGTTC
 651  TGTCTCGGCG CGTCTGCGTC TGGCTGGCTG GCATAAATAT CTCACTCGCA
 701  ATCAAATTCA GCCGATAGCG GAACGGGAAG GCGACTGGAG TGCCATGTCC
 751  GGTTTTCAAC AAACCATGCA AATGCTGAAT GAGGGCATCG TTCCCACTGC
 801  GATGCTGGTT GCCAACGATC AGATGGCGCT GGGCGCAATG CGCGCCATTA
 851  CCGAGTCCGG GCTGCGCGTT GGTGCGGATA TCTCGGTAGT GGGATACGAC
 901  GATACCGAAG ACAGCTCATG TTATATCCCG CCGTCAACCA CCATCAAACA
 951  GGATTTTCGC CTGCTGGGGC AAACCAGCGT GGACCGCTTG CTGCAACTCT
1001  CTCAGGGCCA GGCGGTGAAG GGCAATCAGC TGTTGCCCGT CTCACTGGTG
1051  AAAAGAAAAA CCACCCTGGC GC'CCAATACG CAAACCGCCT CTCCCCGCGC
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Table 1 continued

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1101  GTTGGCCGAT TCATTAATGC AACTCGCACG ACAGGTCTCG AGACTGGAAA

                stop codon              start codon
                lac I                   lac Z                    (1)
1151  GCGGGCAGTG AGCGCTAGGA GGTAACTTAT GGAA'AGCTTG GCACTGGCCG
1201  TCGTTTTACA ACGTCGTGAC TGGGAAAACC CTGGCGTTAC CCAACTTAAT
1251  CGCCTTGCAG CACATCCCCC TTTCGCCAGC TGGCGTAATA GCGAAGAGGC
1301  CCGCACCGAT CGCCCTTCCC AACAGTTGCG CAGCCTGAAT GGCGAATGGC
1351  GCTTTGCCTG GTTTCCGGCA CCAGAAGCGG TGCCGGAAAG CTGGCTGGAG
1401  TGCGATCTTC CTGAGGCCGA TACTGTCGTC GTCCCCTCAA ACTGGCAGAT
1451  GCACGGTTAC GATGCGCCCA TCTACACCAA CGTAACCTAT CCCATTACGG
1501  TCAATCCGCC GTTTGTTCCC ACGGAGAATC CGACGGGTTG TTACTCGCTC
1551  ACATTTAATG TTGATGAAAG CTGGCTACAG GAAGGCCAGA CGCGAATTAT
1601  TTTTGATGGC GTTAACTCGG CGTTTCATCT GTGGTGCAAC GGGCGCTGGG
1651  TCGGTTACGG CCAGGACAGT CGTTTGCCGT CTGAATTTGA CCTGAGCGCA
1701  TTTTTACGCG CCGGAGAAAA CCGCCTCGCG GTGATGGTGC TGCGTTGGAG
1751  TGACGGCAGT TATCTGGAAG ATCAGGATAT GTGGCGGATG AGCGGCATTT
1801  TCCGTGACGT CTCGTTGCTG CATAAACCGA CTACACAAAT CAGCGATTTC
1851  CATGTTGCCA CTCGCTTTAA TGATGATTTC AGCCGCGCTG TACTGGAGGC
1901  TGAAGTTCAG ATGTGCGGCG AGTTGCGTGA CTACCTACGG GTAACAGTTT
1951  CTTTATGGCA GGGTGAAACG CAGGTCGCCA GCGGCACCGC GCCTTTCGGC
2001  GGTGAAATTA TCGATGAGCG TGGTGGTTAT GCCGATCGCG TCACACTACG
2051  TCTGAACGTC GAAAACCCGA AACTGTGGAG CGCCGAAATC CCGAATCTCT
2101  ATCGTGCGGT GGTTGAACTG CACACCGCCG ACGGCACGCT GATTGAAGCA
2151  GAAGCCTGCG ATGTCGGTTT CCGCGAGGTG CGGATTGAAA ATGGTCTGCT
2201  GCTGCTGAAC GGCAAGCCGT TGCTGATTCG AGGCGTTAAC CGTCACGAGC
2251  ATCATCCTCT GCATGGTCAG GTCATGGATG AGCAGACGAT GGTGCAGGAT
2301  ATCCTGCTGA TGAAGCAGAA CAACTTTAAC GCCGTGCGCT GTTCGCATTA
2351  TCCGAACCAT CCGCTGTGGT ACACGCTGTG CGACCGCTAC GGCCTGTATG
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Table 1 continued

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2401  TGGTGGATGA AGCCAATATT GAAACCCACG GCATGGTGCC AATGAATCGT
2451  CTGACCGATG ATCCGCGCTG GCTACCGGCG ATGAGCGAAC GCGTAACGCG
2501  AATGGTGCAG CGCGATCGTA ATCACCCGAG TGTGATCATC TGGTCGCTGG
2551  GGAATGAATC AGGCCACGGC GCTAATCACG ACGCGCTGTA TCGCTGGATC
2601  AAATCTGTCG ATCCTTCCCG CCCGGTGCAG TATGAAGGCG GCGGAGCCGA
2651  CACCACGGCC ACCGATATTA TTTGCCCGAT GTACGCGCGC GTGGATGAAG
2701  ACCAGCCCTT CCCGGCTGTG CCGAAATGGT CCATCAAAAA ATGGCTTTCG
2751  CTACCTGGAG AGACGCGCCC GCTGATCCTT TGCGAATACG CCCACGCGAT
2801  GGGTAACAGT CTTGGCGGTT TCGCTAAATA CTGGCAGGCG TTTCGTCAGT
2851  ATCCCCGTTT ACAGGGCGGC TTCGTCTGGG ACTGGGTGGA TCAGTCGCTG
2901  ATTAAATATG ATGAAAACGG CAACCCGTGG TCGGCTTACG GCGGTGATTT
2951  TGGCGATACG CCGAACGATC GCCAGTTCTG TATGAACGGT CTGGTCTTTG
3001  CCGACCGCAC GCCGCATCCA GCGCTGACGG AAGCAAAACA CCAGCAGCAG
3051  TTTTTCCAGT TCCGTTTATC CGGGCAAACC ATCGAAGTGA CCAGCGAATA
3101  CCTGTTCCGT CATAGCGATA ACGAGCTCCT GCACTGGATG GTGGCGCTGG
3151  ATGGTAAGCC GCTGGCAAGC GGTGAAGTGC CTCTGGATGT CGCTCCACAA
3201  GGTAAACAGT TGATTGAACT GCCTGAACTA CCGCAGCCGG AGAGCGCCGG
3251  GCAACTCTGG CTCACAGTAC GCGTAGTGCA ACCGAACGCG ACCGCATGGT
3301  CAGAAGCCGG GCACATCAGC GCCTGGCAGC AGTGGCGTCT GGCGGAAAAC
3351  CTCAGTGTGA CGCTCCCCGC CGCGTCCCAC GCCATCCCGC ATCTGACCAC
3401  CAGCGAAATG GATTTTTGCA TCGAGCTGGG TAATAAGCGT TGGCAATTTA
3451  ACCGCCAGTC AGGCTTTCTT TCACAGATGT GGATTGGCGA TAAAAAACAA
3501  CTGCTGACGC CGCTGCGCGA TCAGTTCACC CGTGCACCGC TGGATAACGA
3551  CATTGGCGTA AGTGAAGCGA CCCGCATTGA CCCTAACGCC TGGGTCGAAC
3601  GCTGGAAGGC GGCGGGCCAT TACCAGGCCG AAGCAGCGTT GTTGCAGTGC
3651  ACGGCAGATA CACTTGCTGA TGCGGTGCTG ATTACGACCG CTCACGCGTG
3701  GCAGCATCAG GGGAAAACCT TATTTATCAG CCGGAAAACC TACCGGATTG
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Table 1 continued

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3751  ATGGTAGTGG TCAAATGGCG ATTACCGTTG ATGTTGAAGT GGCGAGCGAT
3801  ACACCGCATC CGGCGCGGAT TGGCCTGAAC TGCCAGCTGG CGCAGGTAGC
3851  AGAGCGGGTA AACTGGCTCG GATTAGGGCC GCAAGAAAAC TATCCCGACC
3901  GCCTTACTGC CGCCTGTTTT GACCGCTGGG ATCTGCCATT GTCAGACATG
3951  TATACCCCGT ACGTCTTCCC GAGCGAAAAC GGTCTGCGCT GCGGGACGCG
4001  CGAATTGAAT TATGGCCCAC ACCAGTGGCG CGGCGACTTC CAGTTCAACA
4051  TCAGCCGCTA CAGTCAACAG CAACTGATGG AAACCAGCCA TCGCCATCTG
4101  CTGCACGCGG AAGAAGGCAC ATGGCTGAAT ATCGACGGTT TCCATATGGG
                                                  mutated EcoRI
                                                  site
4151  GATTGGTGGC GACGACTCCT GGAGCCCGTC AGTATCGGCG NNNNNNCAGC
                                                  stop codon
                                                  lac Z
4201  TGAGCGCCGG TCGCTACCAT TACCAGTTGG TCTGGTGTCA AAAATAATAA
                                                  start codon
                                                       lac Y
4251  TAACCGGGCA GGCCATGTCT GCCCGTATTT CGCGTAAGGA AATCCATTAT
4301  GTACTATTTA AAAAACACAA ACTTTTGGAT GTTCGGTTTA TTCTTTTTCT
4351  TTTACTTTTT TATCATGGGA GCCTACTTCC CGTTTTTCCC GATTTGGCTA
4401  CATGACATCA ACCATATCAG CAAAAGTGAT ACGGGTATTA TTTTTGCCGC
4451  TATTTCTCTG TTCTCGCTAT TATTCCAACC GCTGTTTGGT CTGCTTTCTG
4501  ACAAACTCGG GCTGCGCAAA TACCTGCTGT GGATTATTAC CGGCATGTTA
4551  GTGATGTTTG CGCCGTTCTT TATTTTTATC TTCGGGCCAC TGTTACAATA
4601  CAACATTTTA GTAGGATCGA TTGTTGGTGG TATTTATCTA GGCTTTTGTT
4651  TTAACGCCGG TGCGCCAGCA GTAGAGGCAT TTATTGAGAA AGTCAGCCGT
4701  CGCAGTAATT TCGAATTTGG TCGCGCGCGG ATGTTTGGCT GTGTTGGCTG
4751  GGCGCTGTGT GCCTCGATTG TCGGCATCAT GTTCACCATC AATAATCAGT
4801  TTGTTTTCTG GCTGGGCTCT GGCTGTGCAC TCATCCTCGC CGTTTTACTC
4851  TTTTTCGCCA AAACGGATGC GCCCTCTTCT GCCACGGTTG CCAATGCGGT
4901  AGGTGCCAAC CATTCGGCAT TTAGCCTTAA GCTGGCACTG GAACTGTTCA
```

Table 1 continued

```
4951  GACAGCCAAA ACTGTGGTTT TTGTCACTGT ATGTTATTGG CGTTTCCTGC
5001  ACCTACGATG TTTTTGACCA ACAGTTTGCT AATTTCTTTA CTTCGTTCTT
5051  TGCTACCGGT GAACAGGGTA CGCGGGTATT TGGCTACGTA ACGACAATGG
5101  GCGAATTACT TAACGCCTCG ATTATGTTCT TTGCGCCACT GATCATTAAT
5151  CGCATCGGTG GGAAAAACGC CCTGCTGCTG GCTGGCACTA TTATGTCTGT
5201  ACGTATTATT GGCTCATCGT TCGCCACCTC AGCGCTGGAA GTGGTTATTC
5251  TGAAAACGCT GCATATGTTT GAAGTACCGT TCCTGCTGGT GGGCTGCTTT
5301  AAATATATTA CCAGCCAGTT TGAAGTGCGT TTTTCAGCGA CGATTTATCT
5351  GGTCTGTTTC TGCTTCTTTA AGCAACTGGC GATGATTTTT ATGTCTGTAC
5401  TGGCGGGCAA TATGTATGAA AGCATCGGTT TCCAGGGCGC TTATCTGGTG
5451  CTGGGTCTGG TGGCGCTGGG CTTCACCTTA ATTTCCGTGT TCACGCTTAG

                                                  stop codon
                                                      lac Y
5501  CGGCCCCGGC CCGCTTTCCC TGCTGCGTCG TCAGGTGAAT GAAGTCGCTT
5551  AAGCAATCAA TGTCGGATGC GGCGCGACGC TTATCCGACC AACATATCAT

                      start codon
                          lac A
5601  AACGGAGTGA TCGCATTGAA CATGCCAATG ACCGAAAGAA TAAGAGCAGG
5651  CAAGCTATTT ACCGATATGT GCGAAGGCTT ACCGGAAAAA AGACTTCGTG
5701  GGAAAACGTT AATGTATGAG TTTAATCACT CGCATCCATC AGAAGTTGAA
5751  AAAAGAGAAA GCCTGATTAA AGAAATGTTT GCCACGGTAG GGGAAAACGC
5801  CTGGGTAGAA CCGCCTGTCT ATTTCTCTTA CGGTTCCAAC ATCCATATAG
5851  GCCGCAATTT TTATGCAAAT TTCAATTTAA CCATTGTCGA TGACTACACG
5901  GTAACAATCG GTGATAACGT ACTGATTGCA CCCAACGTTA CTCTTTCCGT
5951  TACGGGACAC CCTGTACACC ATGAATTGAG AAAAAACGGC GAGATGTACT
6001  CTTTTCCGAT AACGATTGGC AATAACGTCT GGATCGGAAG TCATGTGGTT
6051  ATTAATCCAG GCGTCACCAT CGGGGATAAT TCTGTTATTG GCGCGGGTAG
6101  TATCGTCACA AAAGACATTC CACCAAACGT CGTGGCGGCT GGCGTTCCTT
6151  GTCGGGTTAT TCGCGAAATA AACGACCGGG ATAAGCACTA TTATTTCAAA
```

Table 1 continued

stop codon
lac A

```
6201  GATTATAAAG TTGAATCGTC AGTTTAAATT ATAAAAATTG CCTGATACGC
6251  TGCGCTTATC AGGCCTACAA GTTCAGCGAT CTACATTAGC CGCATCCGGC
6301  ATGAACAAAG CGCAGGAACA AGCGTCGCAT CATGCCTCTT TGACCCACAG
6351  CTGCGGAAAA CGTACTGGTG CAAAACGCAG GGTTATGATC ATCAGCCCAA
6401  CGACGCACAG CGCATGAAAT GCCCAGTCCA TCAGGTAATT GCCGCTGATA
6451  CTACGCAGCA CGCCAGAAAA CCACGGGGCA AGCCCGGCGA TGATAAAACC
6501  GATTCCCTGC ATAAACGCCA CCAGCTTGCC AGCAATAGCC GGTTGCACAG
6551  AGTGATCGAG CGCCAGCAGC AAACAGAGCG GAAACGCGCC GCCCAGACCT
6601  AACCCACACA CCATCGCCCA CAATACCGGC AATTGCATCG GCAGCCAGAT
6651  AAAGCCGCAG AACCCCACCA GTTGTAACAC CAGCGCCAGC ATTAACAGTT
6701  TGCGCCGATC CTGATGGCGA GCCATAGCAG GCATCAGCAA AGCTCCTGCG
6751  GCTTGCCCAA GCGTCATCAA TGCCAGTAAG GAACCGCTGT ACTGCGCGCT
6801  GGCACCAATC TCAATATAGA AAGCGGGTAA CCAGGCAATC AGGCTGGCGT
6851  AACCGCCGTT AATCAGACCG AAGTAAACAC CCAGCGTCCA CGCGCGGGGA
6901  GTGAATACCA CGCGAACCGG AGTGGTTGTT GTCTTGTGGG AAGAGGCGAC
6951  CTCGCGGGCG CTTTGCCACC ACCAGGCAAA GAGCGCAACA ACGGCAGGCA
7001  GCGCCACCAG GCGAGTGTTT GATACCAGGT TTCGCTATGT TGAACTAACC
7051  AGGGCGTTAT GGCGGCACCA AGCCCACCGC CGCCCATCAG AGCCGCGGAC
7101  CACAGCCCCA TCACCAGTGG CGTGCGCTGC TGAAACCGCC GTTTAATCAC
7151  CGAAGCATCA CCGCCTGAAT GATGCCGATC CCCACCCCAC CAAGCAGTGC
7201  GCTGCTAAGC AGCAGCGCAC TTTGCGGGTA AAGCTCACGC ATCAATGCAC
7251  CGACGGCAAT CAGCAACAGA CTGATGGCGA CACTGCGACG TTCGCTGACA
7301  TGCTGATGAA GCCAGCTTCC GGCCAGCGCC AGCCCGCCCA TGGTAACCAC

                SalI site in pSKS107
7351  CGGCAGAGCG GTCGAC
```

(1) sequence in bold, between the two ' marks, is synthetic DNA used to fuse the lac operon to the lac I or lac I^Q gene operon.

(2) pSKS107 contains an unsequenced mutation at the EcoRI site normally found in the lac Z gene.

Table 2.

Lactose Inducibility of Various Constructs			
Hours after[1] Induction	Lactose (mM)	Cells/ml	Toxin[2] (ug/ml)
MR471			
40	0	1.6×10^{10}	none detected
40 no lactose	2 mM IPTG	2.3×10^{10}	1003
15	20	9.1×10^{9}	1011
24	20	1.2×10^{10}	737
15	40	1.8×10^{10}	887
24	40	1.1×10^{10}	1025
23[3]	8.3[4]	1.9×10^{10}	938
39[3]	8.3[4]	2.0×10^{10}	1555
MB101 containing pMYC485			
24	40	-	775
24	0	-	none detected
MB101 containing pMYC1161			
24	40	-	300
24	0	-	none detected

(1) Cultures were induced upon reaching stationary phase.
(2) Toxin concentration was determined by laser densitometry of Coomassie-stained protein bands after electrophoresis of disrupted cells on SDS-PAGE (LKB Instructional Manual 2222-010).
(3) This experiment used MR471 grown in a 10L fermentor. All other experimental data were generated using same medium in 250 ml baffled shake flasks.
(4) In the fermentor, 8.3 mM lactose/hour was fed into the culture. It was found that MR471 did not metabolize this lactose level in a fermentor, resulting in increased concentrations during the experiment. The experiments done in hake flasks were given a single dose of lactose or IPTG at the indicated times.

Claims

1. A modified lac operon, wherein the CAP binding site for catabolite repression and the promoter/operator are deleted, and wherein the lac I gene is fused to the ZY or ZYA gene, which substantially retains the parent operon's ability to control expression of a heterologous gene.
2. An operon according to claim 1, having the sequence shown in Table 1, or a mutation thereof.
3. A DNA construct comprising an operon according to claim 1 or claim 2 and also the heterologous gene.
4. A transfer vector, e.g. a plasmid, comprising an operon or DNA as defined in any preceding claim.
5. A microbial host transformed by the transfer vector of claim 4.
6. A process for preparing a polypeptide encoded by the heterologous gene, which comprises culturing a microbe comprising DNA as defined in claim 3.

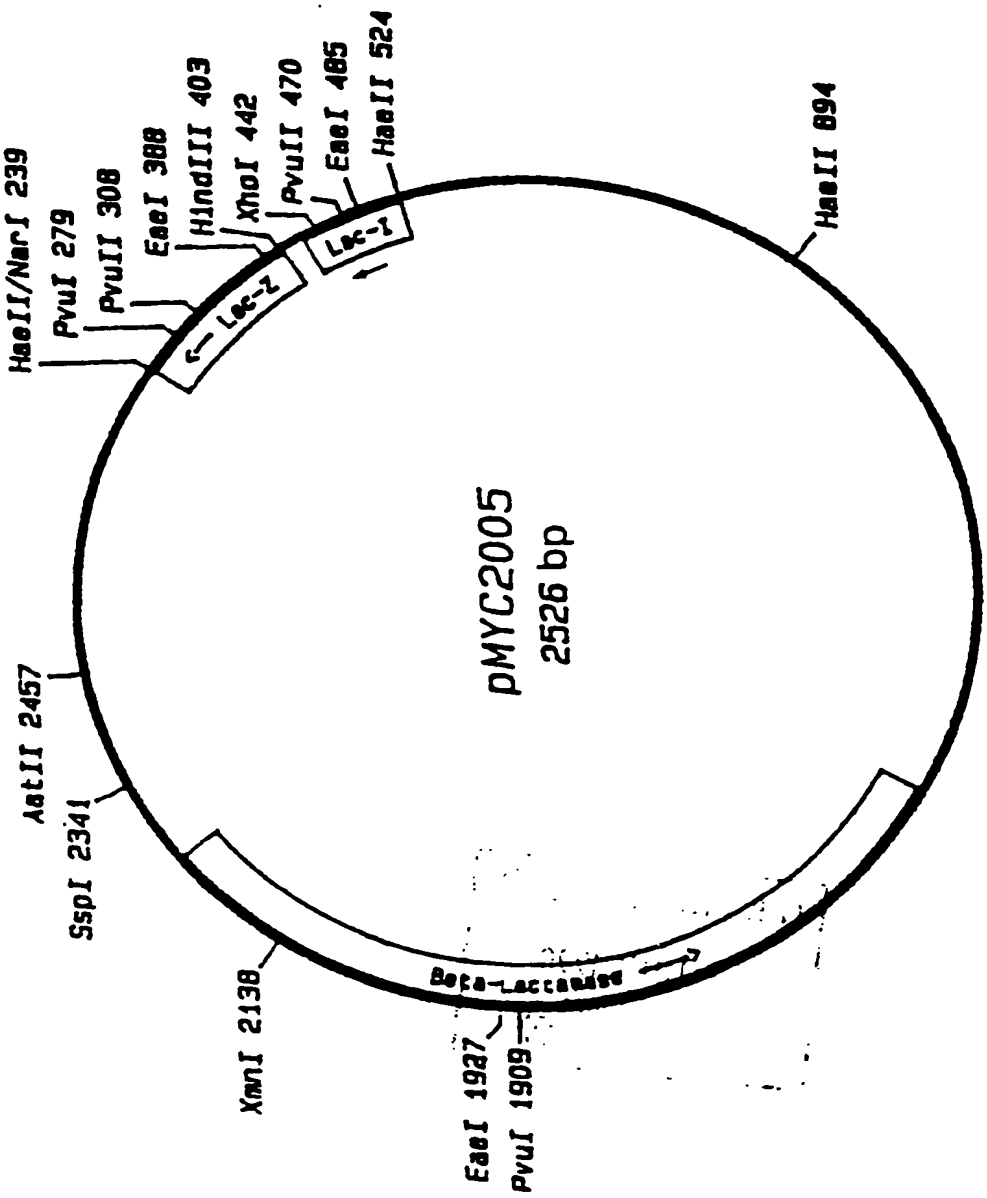

Figure 1

PLASMIDMAP of: pMYC2005 check: 4484 from: 1 to: 2526

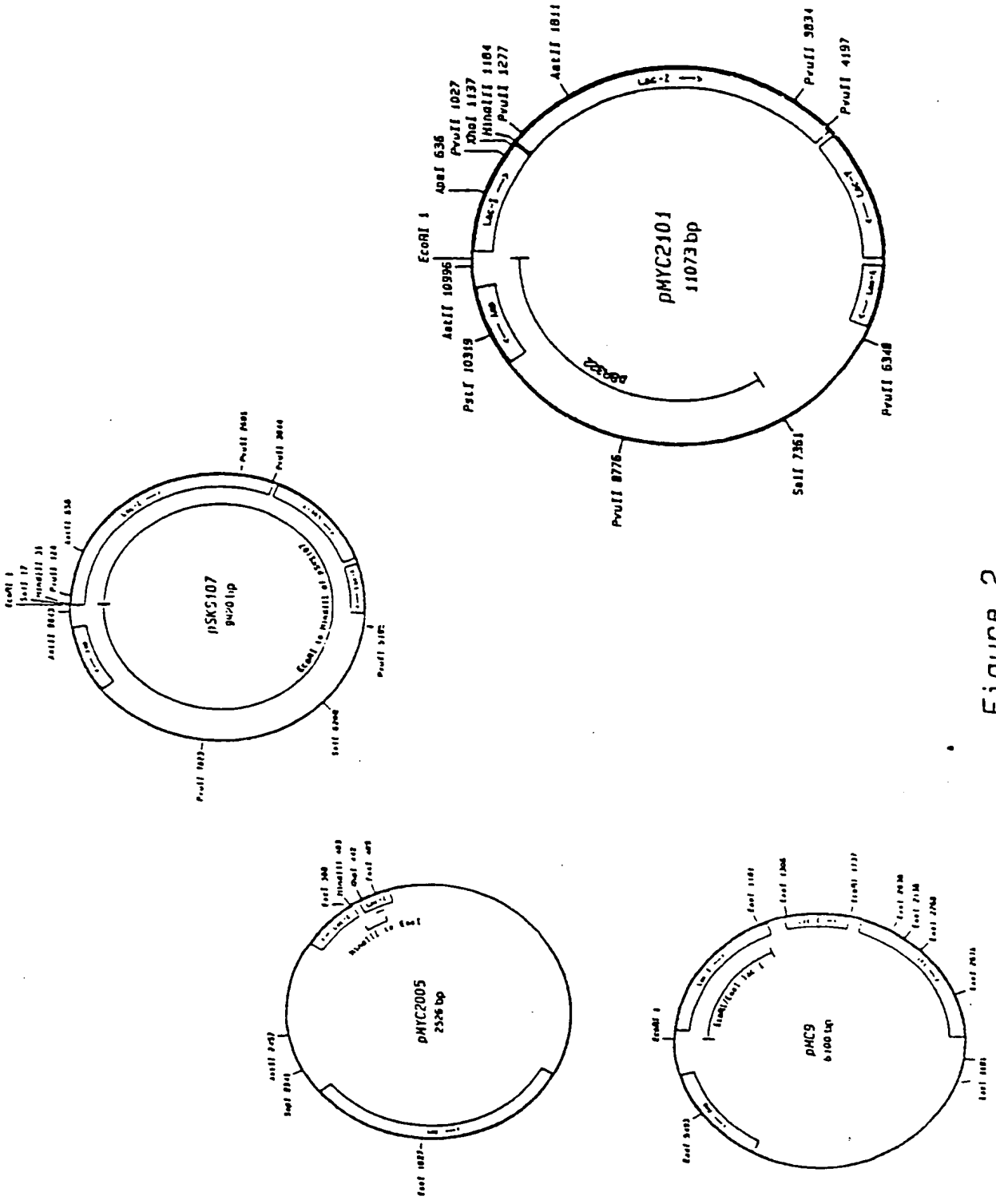

Figure 2

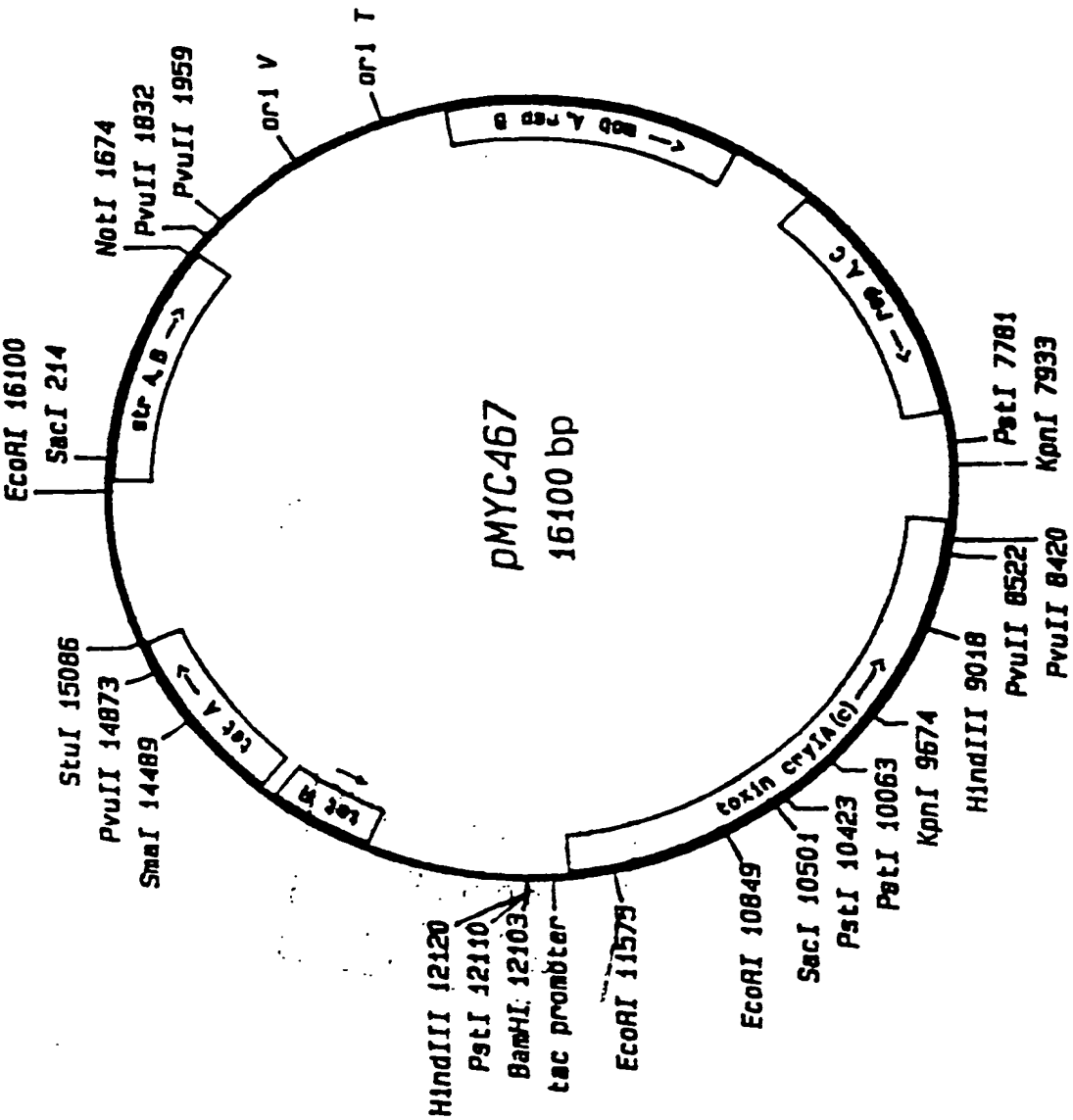

Figure 3

PLASMIDMAP of: pMYC467 check: 3500 from: 1 to: 16100

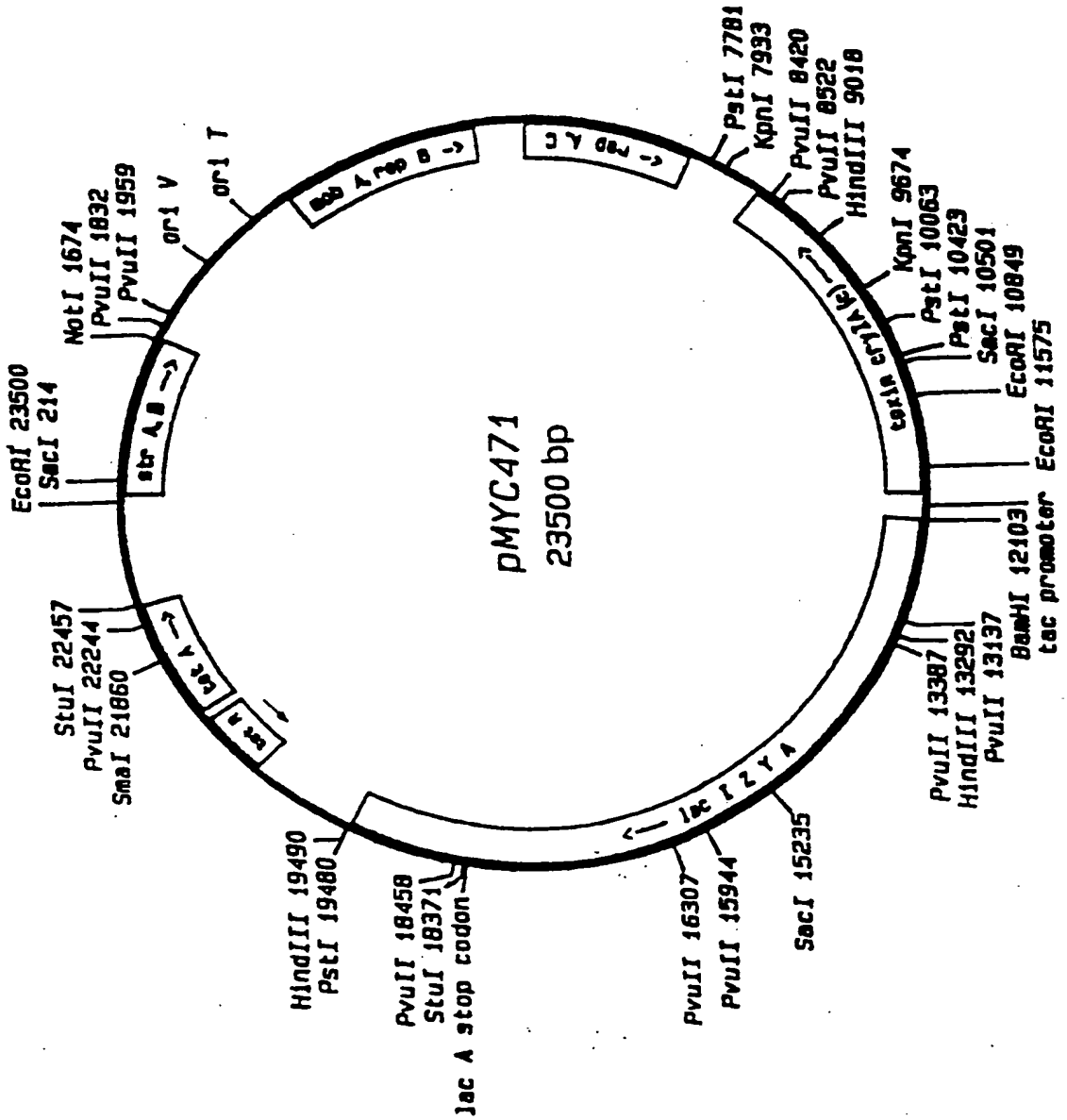

Figure 4

PLASMIDMAP of: pMYC471 check: 3500 from 1 to: 23500

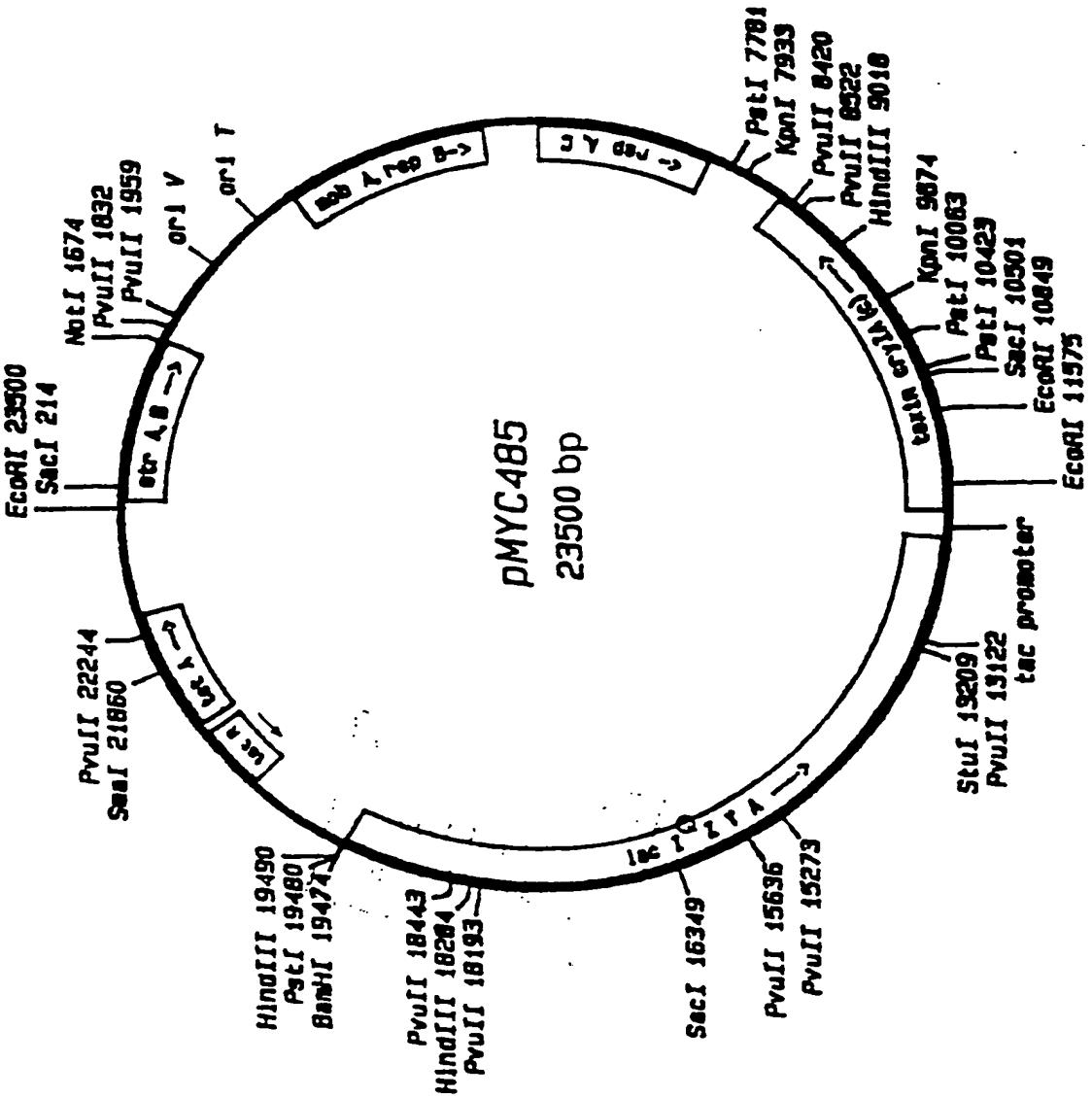

Figure 5

PLASMIDMAP of: pMYC485 check: 3500 from: 1 to: 23500

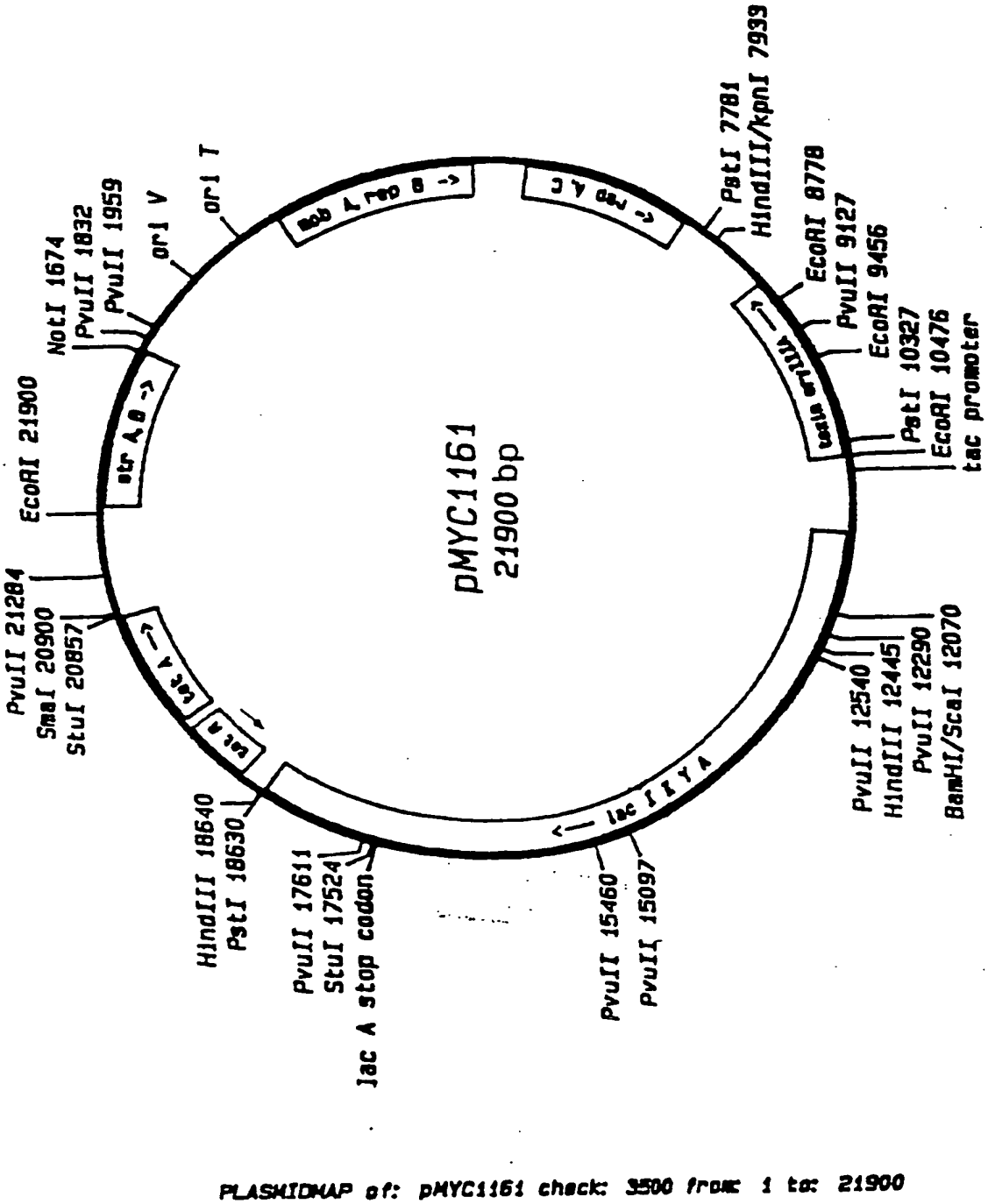

Figure 6

PLASMIDMAP of: pMYC1161 check: 3500 from: 1 to: 21900

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 30 7952

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	GENES & DEVELOPMENT, vol. 1, no. 3, 1987, pages 227-237, New York, US; J.K. WHORISKEY et al.: "Genetic rearrangements and gene amplification in Escherichia coli: DNA sequences at the junctures of amplified gene fusions" * Abstract; page 228, column 2, line 26 - page 229, column 2, line 24; page 229, figure 3 *	1,2	C 12 N 15/72 C 12 N 1/21
A	JOURNAL OF MOLECULAR BIOLOGY, vol. 186, no. 4, 1985, pages 733-742, London, GB; K.C. CONE et al.: "Functional analysis of lac repressor restart sites in translational initiation and reinitiation" * Abstract; page 735, column 1, line 40 - page 736, column 1, line 8; page 739, column 2, line 28 - page 740, column 1, line 13 *	1,2	
A	NUCLEIC ACIDS RESEARCH, vol. 12, no. 13, 1984, pages 5449-5464, Oxford, GB; X.-M. YU et al.: "Deletion analysis of the CAP-cAMP binding site of the Escherichia coli lactose promoter" * Abstract; page 84, line 1 - page 85, line 5 *	1	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C 12 N 15/00

The present search report has been drawn up for all claims

Place of search	Date of completion of search	Examiner
The Hague	17 October 90	GURDJIAN D P M

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